

**MOLECULES, COMPOSITIONS, METHODS AND KITS FOR
APPLICATIONS ASSOCIATED WITH FLAVIVIRUSES**

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Cross-reference to related applications

[0001] This application claims the benefit of the prior filing date of U.S. provisional patent application number 60/442,517, filed January 22, 2003, entitled "Interaction between cell receptor protein and flavivirus attachment domain/s and uses thereof" by Dr. Mary Ng Mah Lee and Justin Chu Jang Hann, the disclosure of which is incorporated herein by reference in its entirety.

Field of the disclosure

[0002] The present disclosure relates to the field of virology, and in particular to molecules, compositions, methods and kits for applications associated with flaviviruses.

Background of the disclosure

[0003] The family *Flaviviridae* contains at least 70 arthropod-transmitted viruses, many of which infect humans and other vertebrates. A subgroup of the *Flaviviridae* family, the Japanese encephalitis serocomplex, includes West Nile Virus, St. Louis encephalitis, Murray Valley encephalitis and kunjin viruses. West Nile virus, in particular, is most commonly found in Africa and the Middle East.

[0004] All flaviviruses, including West Nile Virus, St Louis encephalitis, dengue, Japanese encephalitis, yellow fever and kunjin viruses share similar size, symmetry and appearance. Despite the fact that flaviviruses may use different process to enter a host cell, such as endocytosis (described for West Nile Virus and Kunjin Virus) and direct

fusion of the cell (described for dengue and Encephalitis Virus), entry of all flaviviruses into the host-cell involves an interaction between the virus and a receptor of the cell.

[0005] Several studies have shown that the viral envelope protein of flaviviruses plays a crucial role in mediating virus-host cellular receptor interaction. Based on crystallography data of tick-borne encephalitis flavivirus viral envelope protein, Rey and colleagues (1995) noted that each viral envelope protein monomer is folded into 3 distinct domains. A central domain I is the antigenic domain that carries the N-glycosylation site. Domain II of the viral envelope protein is believed to be responsible for pH-dependent fusion of the viral envelope protein to the endosomal membrane during uncoating, and domain III is important for flavivirus binding to host cells.

[0006] With reference to West Nile virus, Beasley and Barrett (2002) focused on the importance of subportions of the Domain III in West Nile virology. They identified and mapped as epitopes, portions of Domain III whose neutralization by single monoclonal antibodies may result in neutralization of the virus.

[0007] The specific interaction between flaviviruses and a vertebrate cell surface or surface membrane receptor is unknown. Without knowledge of the details of this interaction, it has proven difficult to specifically treat or prevent the disease. Therefore, there is clearly a need for the identification of the cell receptor, as well as the domain/s of the flavivirus that mediate their respective interactions.

Summary Of The Disclosure

[0008] The present disclosure overcomes the problems and disadvantages of the prior art.

[0009] According to a first aspect of the present disclosure, a method for controlling a flavivirus entry into a cell is disclosed, comprising administering to the cell an agent functionally interfering with a flavivirus receptor protein, the receptor protein being an integrin.

[0010] The integrin preferably comprises integrin subunit $\beta 3$ or integrin subunit αV , and most preferably is an $\alpha V\beta 3$ integrin. The agent functionally interfering with a flavivirus receptor protein is preferably a functional blocking antibody against the

integrin, or a competitive ligand for the integrin, in particular an RGD peptide or a natural ligand selected from the group consisting of fibronectin, vitronectin, laminin and chondroitin.

[0011] According to a second aspect, a method for controlling flavivirus entry into a cell is disclosed, comprising administering to the cell an agent interfering with the expression of a flavivirus receptor protein, the receptor protein being integrin.

[0012] The agent interfering with the expression of the flavivirus receptor protein is preferably a siRNA against the integrin.

[0013] According to a third aspect, a kit for controlling flavivirus entry into a cell is disclosed, comprising: the flavivirus; an agent functionally interfering with an integrin. The flavivirus and the agent are to be used in the method disclosed herein.

[0014] Preferably, the agent functionally interfering with an integrin is a functional blocking antibody against the integrin or a competitive ligand for the integrin.

[0015] According to a fourth aspect, a further kit for controlling flavivirus entry into a cell, is disclosed, comprising: the flavivirus; and an agent interfering with expression of an integrin. The flavivirus and the agent interfering with the expression of the integrin are to be used according to the method disclosed herein.

[0016] An agent functionally interfering with an integrin may also be included in the kit disclosed herein and is to be used according to the method comprising its administration disclosed herein. The agent interfering with the expression of an integrin is preferably an SiRNA against the integrin.

[0017] According to a fifth aspect, a further method for controlling a flavivirus entry into a cell is disclosed, comprising administering to the cell an agent functionally interfering with an ATPase in the plasma membrane of the cell, preferably a functionally blocking antibody against the ATPase.

[0018] According to sixth aspect, a kit for controlling a flavivirus entry into a cell is disclosed, comprising: the flavivirus; and an agent functionally interfering with an ATPase located in the plasma membrane of the cell. The flavivirus and the agent are to be used according to the method disclosed herein.

[0019] An agent functionally interfering with an integrin and/or an agent interfering with the expression of an integrin may also be included in the kit and are to be used according to the methods comprising the respective administration herein also disclosed.

[0020] According to a seventh aspect, a method for controlling a flavivirus entry into a cell is also disclosed, which comprises administering to the cell an agent functionally interfering with a flavivirus receptor protein, the receptor protein being a neurotensin receptor.

[0021] Preferably, the agent functionally interfering with a flavivirus receptor protein is a functional blocking antibody against the neurotensin receptor, or a competitive ligand for the neurotensin receptor, in particular neurotensin.

[0022] According to an eighth aspect, a kit for controlling a flavivirus entry into a cell is disclosed, comprising: the flavivirus; and an agent functionally interfering with a neurotensin receptor in the cell. The flavivirus and the agent are to be used according to the method disclosed herein.

[0023] An agent functionally interfering with an integrin, an agent interfering with the expression of an integrin and/or an agent functionally interfering with an ATPase in the plasma membrane of the cell may also be included in the kit and are to be used according to the methods comprising the respective administration herein also disclosed.

[0024] According to a ninth aspect, a method for controlling a flavivirus entry into a cell is disclosed, the flavivirus exhibiting a flavivirus envelope protein, the flavivirus envelope protein comprising a domain III, the method comprising administering to the cell an agent functionally interfering with the domain III of the flavivirus envelope protein. Preferably the domain III of the virus comprise a portion having a sequence substantially homologous to SEQ ID NO: 19 or SEQ ID NO: 21.

[0025] According to a further aspect, a method for treating a flavivirus infection in a vertebrate is disclosed, the flavivirus exhibiting a flavivirus envelope protein, the flavivirus envelope protein comprising a domain III. The method comprises administering to the vertebrate a pharmaceutically effective amount of an agent

functionally interfering with the domain III of the envelope protein of the flavivirus, able to inhibit the entry of the flavivirus in the cell.

[0026] According to a further aspect, a pharmaceutical composition for the treatment of a flavivirus infection in a vertebrate is disclosed, the flavivirus exhibiting an envelope protein comprising a domain III. The pharmaceutical composition comprises a pharmaceutically effective amount of an agent interfering with the domain III of the envelope protein able to inhibit the entry of the Flavivirus in the host cell and a pharmaceutically acceptable carrier, vehicle or auxiliary agent.

[0027] Both in the method of treating and pharmaceutical composition, the agent is preferably one of the functionally interfering agent able to inhibit the entry in the cell mentioned above. In particular, a functional blocking antibody against the domain III, preferably a polyclonal antibody, an integrin protein, preferably comprising one or both of the subunits αV and $\beta 3$, or a neurotensin receptor protein or an ATPase, preferably an F-ATPase or V-ATPase, or portions thereof may be used.

[0028] According to a further aspect, a method for inducing immunity to a flavivirus in a vertebrate susceptible to the infection of the flavivirus is disclosed, the flavivirus exhibiting an envelope protein comprising a domain III. The method comprises administering to the vertebrate an immunogenic amount of a polypeptide comprising the domain III, of the envelope protein of the flavivirus, preferably comprising a portion substantially homologous to SEQ ID NO: 19 or SEQ ID NO: 21.

[0029] According to a further aspect, a vaccine for a flavivirus, the flavivirus exhibiting an envelope protein comprising a domain III, is disclosed. The vaccine comprises as an active agent a polypeptide comprising the domain III of the envelope protein of the flavivirus.

[0030] According to a further aspect, a method for diagnosing a flavivirus infection in a vertebrate susceptible to infection by the flavivirus is disclosed, comprising contacting a sample tissue from the vertebrate, with an integrin or neurotensin protein associated with an identifier; and detecting presence or absence of a flavivirus-integrin complex or flavivirus-neurotensin complex by detecting presence of the identifier.

[0031] According to a further aspect, a kit for the diagnosis of flavivirus infection in a vertebrate, susceptible to be infected with the flavivirus, the flavivirus exhibiting an envelope protein comprising domain III is disclosed. The kit comprises at least one agent able to bind the domain III, associated with an identifier, and one or more reagents able to detect the identifier. The agent able to bind domain III and the reagents are to be used according to the diagnostic method disclosed above .

[0032] According to a further aspect, a diagnostic method to analyze a cell susceptibility to flavivirus infection, is disclosed, comprising contacting the cell with an identifier for the presence or expression of an integrin, neurotensin receptor and/or ATP-ase and detecting the presence of the identifier associated to presence or expression of an integrin, neurotensin receptor and/or ATP-ase in the cell.

[0033] According to another aspect, a kit to analyze cell susceptibility to flavivirus infection is disclosed comprising an identifier for the presence or expression of an integrin, neurotensin receptor and or ATP-ase, and a reagent able to detect the presence of the identifier; the identifier and the reagent to be used in the method disclosed above.

[0034] According to a further aspect, an isolated and purified plasma membrane polypeptide of approximately 105KDa comprising a sequence substantially homologous to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5 is disclosed.

[0035] In the methods, kits, composition and vaccine disclosed herein, the flavivirus is preferably a member of the Japanese encephalitis serocomplex, in particular West Nile Virus, and the vertebrate is preferably a mammal, in particular a human being.

Brief Description of the Figures

[0036] In the present disclosure, reference will be made to the enclosed Figures, which provide non-limiting examples of the inventive compositions, method and kits devised by the inventors.

[0037] Figure 1A shows a diagram reporting the effect of phospholipases and proteases treatment on West Nile virus binding molecules present on the surface of

intact Vero cells. The y-axis shows the inhibition of West Nile virus entry expressed as the number of log unit inhibition with respect to untreated samples. On the x-axis, the dosage of the substance administered is reported. PLA= Phospholipase A₂, PLC=Phospholipase C, PLD=Phospholipase D

[0038] Figure 1B shows a diagram reporting the effect of enzyme, sodium periodate and lectin treatment on West Nile virus binding molecules present on the surface of intact Vero cells. The y-axis shows the inhibition of West Nile virus entry expressed as the number of log unit inhibition with respect to untreated samples. On the x-axis, the dosage of substance administered is reported. EH=Endoglycosidase H, OG=O-Glycosidase, MA=α-Mannosidase; FU=α-Fucosidase; Hepl=Heparinase I; HepIII=Heparinase III; SP=Sodium Periodate; WGA=Wheat Germ Agglutinin; PHA=phytohemagglutinin; ConA=Concanavalin-A.

[0039] Figure 2A shows the results of a Virus Overlay Protein Binding Assay (VOPBA) performed on plasma membrane proteins extracted from Vero cells before purification under non-denaturing conditions (Lane 1) as compared to those of proteins purified from the supernatants of uninfected cells (Lane 2). Molecular size markers are indicated on the left side of the Figure by arrows.

[0040] Figure 2B shows the results of a VOPBA performed on plasma membrane proteins extracted from N2A cells extracted before purification under non-denaturing conditions (Lane 1) as compared to those of proteins purified from the supernatants of uninfected cells (Lane 2). Molecular size markers are indicated on the left side of the Figure by arrows.

[0041] Figure 3 shows results of a VOPBA performed on plasma membrane proteins from Vero cells and N2A cells after papain treatment (Lane 2 – Vero cells; lane 4 - N2A), as compared to those of the untreated cells (Lane 1 – Vero cells; lane 3 - N2A). Molecular size markers are indicated on the left side of the Figure by arrows.

[0042] Figure 4 shows results of a VOPBA performed on plasma membrane proteins from Vero cells after papain treatment (Lane 2 – after 0 hours; lane 3 – after 2 hours; Lane 4 – after 4 hours), as compared to those of untreated cells (Lane 1) and those of treated cells further subjected to cycloheximide treatment (Lane 2 – after 0 hour; lane 3

– after 2 hours; Lane 4 – after 4 hours). Molecular size markers are indicated on the left side of the Figure by arrows.

[0043] Figure 5A shows results of a VOPBA performed on plasma membrane proteins extracted from Vero cells (Lanes 1 to 4) and N2A cells (Lanes 5 to 8) either left untreated (Lanes 1 and 5) or subjected to α -mannosidase (Lanes 2 and 6), Endoglycosidase H (Lanes 3 and 7) and O-glycosidase (Lanes 4 and 8). A molecular size marker is indicated on the right side of the Figure by an arrow.

[0044] Figure 5B shows results of a VOPBA performed on plasma membrane proteins obtained from Vero cells pretreated with lectins, concanavalin-A [Lane 1 (untreated), Lane 2 (10 μ g/ml) and Lane 3 (100 μ g/ml)] and phytohemagglutinin [Lanes 4 (untreated), Lane 5 (10 μ g/ml) and Lane 6 (100 μ g/ml)]. A molecular size marker is indicated on the right side of the Figure by an arrow.

[0045] Figure 5C shows results of a VOPBA performed on plasma membrane proteins obtained from Vero cells either left untreated (Lane 1) or treated with 0.1 mM (Lane 2), 1 mM (Lane 3) and 10 mM (Lane 4) of sodium periodate. Molecular size markers are indicated on the left side of the Figure by arrows.

[0046] Figure 6 shows results of a VOPBA performed on membrane proteins from Vero cells and N2A cells after β -mercaptoethanol treatment (Lane 2 – Vero cells; lane 4 - N2A) as compared to those of the untreated cells (Lane 1 – Vero cells and Lane 3 – N2A cells). Molecular size markers are indicated on the left side of the Figure by arrows.

[0047] Figure 7 shows results of a Western Blotting performed on membrane proteins from plasma membrane extracts of Vero cells. Incubation of separated membrane proteins with the preimmune serum (Lane 1) and the anti-105-kDa protein polyclonal antibodies at a dilution of 1:500 (Lane 2) was performed. Molecular size markers are indicated on the left side of the Figure by arrows.

[0048] Figure 8A shows Vero cells processed for immunofluorescence and confocal microscopy. The arrows indicate the 105-kDa proteins distributed along the plasma membrane as shown by the red staining.

[0049] Figure 8B shows apical localization of the 105-kDa membrane proteins on Z-section (cross-section) of polarized Vero C1008 epithelial cells. The arrows indicate the 105-kDa membrane proteins at the apical surface of the cells.

[0050] Figure 9 shows Immunogold-labeling of the 105-kDa proteins on cryo-sections of Vero cells. The arrow indicates the binding of West Nile virus to the 105-kDa membrane protein indicated by arrows at the site of virus attachment.

[0051] Figure 10 shows a VOPBA of Vero plasma membrane protein pre-incubated with anti-105 kDa murine antibodies (Lane 1) compared to that of untreated cells (Lane 2). Molecular size markers are indicated on the left side of the Figure by arrows.

[0052] Figure 11A is a diagram showing the inhibition of West Nile Virus binding in Vero cells pre-incubated with functional blocking integrin antibodies. On the x-axis, the concentration of functional blocking integrin antibodies is shown. On the y-axis, the percentage reduction in virus infection is shown. INTB1= Integrin β 1, INTB2= Integrin β 2, INTB31= integrin β 3, INTB32= integrin β 3 subunit, INTB4= integrin β 4, INTB5= integrin β 5, ALPHAV= Integrin α V, ALPHA V/B3= Integrin α V β 3, ALPHA5/B5= Integrin α 5 β 5.

[0053] Figure 11B is a diagram showing the inhibition of West Nile Virus entry in Vero cells pre-incubated with functional blocking integrin antibodies. On the x-axis the concentration of functional blocking integrin antibodies is shown. on the y-axis the percentage reduction in virus infection is shown. INTB1= monoclonal antibodies against Integrin β 1, INTB2= Integrin β 2, INTB31= monoclonal antibodies against integrin beta 3 subunit purchased from Chemicon,USA, INTB32= monoclonal antibodies against integrin beta 3 subunit purchased from Santa Cruz Biotech, USA., INTB4= monoclonal antibodies against integrin β 4, INTB5= monoclonal antibodies against Integrin β 5, ALPHAV= monoclonal antibodies against Integrin α V, ALPHA V/B3= monoclonal antibodies against Integrin α V β 3, ALPHA5/B5= monoclonal antibodies against Integrin α 5 β 5.

[0054] Figure 12 is a diagram showing the inhibition of Japanese Encephalitis Virus entry into Vero cells by functional blocking integrin antibodies. On the x-axis the integrin blocked by the specific anti integrin antibody is shown. On the y-axis the % inhibition in

virus entry is shown. INTB1= monoclonal antibodies against Integrin β 1; INTB2= monoclonal antibodies against Integrin β 2, INTB31= monoclonal antibodies against integrin beta 3 subunit purchased from Chemicon,USA, INTB32= monoclonal antibodies against integrin beta 3 subunit purchased from Santa Cruz Biotech, USA.; INTB4=monoclonal antibodies against integrin β 4; INTB5= monoclonal antibodies against Integrin β 5, ALPHAV= monoclonal antibodies against Integrin α V; ALPHA V/B3= monoclonal antibodies against Integrin α V β 3; ALPHA5/B5= monoclonal antibodies against Integrin α 5 β 5.

[0055] Figure 13 is a diagram showing the effect of divalent cations chelator EDTA on the entry of West Nile Virus (WNV) and Japanese Encephalitis Virus (JEV) in Vero cells. On the x-axis, the concentration of EDTA administered is shown. The y-axis shows percentage reduction of virus infection.

[0056] Figure 14 is a diagram showing a competitive entry study of West Nile Virus (WNV) and Japanese Encephalitis Virus (JEV) with physiological ligands on Vero cells pretreated with such ligands. On the x-axis, the competitive ligands used, are shown. On the y-axis, the percentage reduction of virus infection is shown. RGE1= RGE-peptide; RGD1= RGD-peptide; FIBRO1=Fibronectin; VITRO1= Vitronectin; LAMININ 1= laminin; CHONSUL1=Chondroitin; Heparin= Heparin.

[0057] Figure 15 shows distribution and localization of integrin α V β 3 (A) and 105KDa plasma membrane glycoprotein (B) in Vero cells by immunofluorescence staining.

[0058] Figure 16 shows results of gene silencing of integrin α V β 3 subunits α V (B) and β 3 (D) in Vero cells compared to control for α V (A) and β 3 (C). Presence of the subunits is shown by immunofluorescence staining.

[0059] Figure 17 is a diagram showing the effects of down-regulation of integrin α V and β 3 subunits to the entry of WNV into Vero cells. On x-axis concentration of siRNA used is reported. On y-axis percentage inhibition of WNV entry is reported. INTAlpha V1= Integrin alpha V subunit region 1, INTalpha2= Integrin alpha V subunit region 2, INTB31= Integrin beta 3 subunit region 1, INTB32 = Integrin beta 3 subunit region 2, GAPDH= Glyceraldehyde-3-phosphate dehydrogenase.

[0060] Figure 18 is a diagram showing effects of administration of antibodies against ATPases. On the x-axis, the antibodies against the respective protein are indicated. On the y-axis, the percentage reduction of virus entry is indicated. ATPB1= monoclonal antibodies against plasma membrane ATPase beta subunit, ATPB2= polyclonal antibodies against plasma membrane ATPase beta subunit, ATPA1= monoclonal antibodies against plasma membrane ATPase alpha subunit, ATPA2= polyclonal antibodies against plasma membrane ATPase alpha subunit, CALTYPE1= monoclonal antibodies against calcium dihydropyridine receptor alpha, CALTYPE2= monoclonal antibodies against calcium dihydropyridine receptor beta, VATPASE1= monoclonal antibodies against VATPase E, VATPASE2= monoclonal antibodies against VATPase.

[0061] Figure 19 is a diagram showing blockage of WNV entry by antibodies against neurotensin receptor, in Vero cells (black column) and in A172 neuroblastoma cells (grey column). On the x-axis, the concentration of anti-neurotensin receptor is shown. The y-axis shows the percentage reduction of virus entry.

[0062] Figure 20 is a diagram showing WNV competing binding of neurotensin receptor with its natural ligand in A172 neuroblastoma cells. On the x-axis, the concentration of neurotensin administered before incubation of the cells with WNV is shown. The y-axis shows percentage inhibition of virus entry.

[0063] Figure 21 shows immunofluorescence assays performed in A172 cells (A), and in A172 cells transfected with pSilencer-siRNA neurotensin receptor (B).

[0064] Figure 22 shows results of a Western Blotting carried out with monoclonal antibodies against (A) E-protein of WNV and (B) anti-His lysate, whole cell lysate where domain II of WNV was cloned and expressed as His-tagged fusion protein. (A) lane 1= IBV nucleocapsid protein; lane 2=Dengue infected whole cells lysate; lane 3= buffer; lane 4= DIII protein; and lane 5= West Nile virus infected whole cell lysate. (B) lane 1= IBV nucleocapsid protein; lane 2= buffer; lane 3= DIII protein; and lane 4= West Nile virus infected whole cell lysate. Molecular size markers are indicated on the left side of the Figure by arrows.

[0065] Figure 23 is a diagram showing results of competitive inhibition of WNV and Dengue virus entry with soluble recombinant WNV envelope DIII. On the x-axis,

concentration of inhibitor(s) is reported. On the y-axis, percentage inhibition of virus entry is reported.

[0066] Figure 24 shows production of murine polyclonal antibodies against recombinant DIII protein. Lane 1= recombinant DIII protein with anti-DIII protein polyclonal murine antibodies; and lane 2= DIII protein with pre-immunized murine sera. Molecular size markers are indicated on the left side of the Figure by arrows.

[0067] Figure 25 is a diagram showing results of plaque neutralization of WNV with murine polyclonal antibodies against envelope DIII protein. On the x-axis, various grades of dilution used are reported. On the y-axis, percentage inhibition of virus infection is reported.

Detailed Description of the Disclosure

[0068] A method for controlling the entry of a flavivirus into a cell is described. In particular, the method is based on the identification of integrins as receptors which mediate entry of the flavivirus into the cell.

[0069] In their quality as flavivirus receptors, integrins have been found to surprisingly mediate entry of a wide number of flaviviruses using diverse processes to enter the host cell, such as endocytosis and cell fusion. In particular, integrins have been shown to mediate the entry in the cell of flaviviruses belonging to the Japanese Encephalitis Serocomplex, in particular West Nile Virus, St. Louis encephalitis, Murray Valley encephalitis, as well as the entry of other flaviviruses such as dengue and kunjin.

[0070] Additionally, the activity of integrins as flavivirus receptors disclosed herein applies to a wide range of cell systems, including brain cells, and to a wide number of organisms, including vertebrates and human beings.

[0071] Integrins have been identified to be flavivirus receptors through a series of experiments extensively described in the examples that follow.

[0072] In particular, in a first series of experiments, described in the examples 1 to 9, the receptor has been first identified as a protease sensitive glycoprotein with complex N-linked sugars containing α -mannose residues, localized on the cell membrane. In

particular, a 105-KDa glycoprotein exhibiting all these properties has been isolated (see example 2).

[0073] The experiments have been carried out on Vero cells and N2A cells. Both are cells lines highly permissive for West Nile Virus, which has been used as a model for flaviviruses. Vero cells is a Green Monkey cell line and has been used as a cell system to isolate the receptor for WNV. N2A is a mouse derived brain cell line, which has been used as an alternative to human brain cells, since West Nile virus has shown tropism to brain cells during infection in mammals.

[0074] West Nile virus, in particular the Sarafend strain, has been used in these experiments. West Nile Virus has been used as a representative of the flavivirus and in particular of the flavivirus belonging to the Japanese Encephalitis Serocomplex group. Other flaviviruses of the Japanese Encephalitis Serocomplex group, including kunjin ,as well as flaviviruses not belonging to such group, such as dengue, were also used to extend the analysis on the integrins' activity as flavivirus receptors to the entire flavivirus family.

[0075] The experiments extensively described in examples 10 and 11 confirm the 105KDa glycoprotein's ability to act as a receptor for West Nile and other flaviviruses, in particular those of the Japanese Encephalitis serocomplex group, including St. Louis encephalitis, Murray Valley Encephalitis, as well as dengue and kunjin. In particular in view of those results, a significant receptor activity for any flaviviruses belonging to the flavivirus family is expected. In particular, receptor activity for the flavivirus having an E protein substantially homologous to the E protein of a member of the Japanese Serocomplex group, such as yellow fever and tick borne, is expected.

[0076] Further analysis of the 105KDa protein has confirmed location of the receptor on the membrane (example 12) and the fact that the receptor is an integrin (example 13). In particular, further experiments assaying flavivirus entry inhibition, sequencing of the 105KDa protein and Virus Overlay Protein Binding Assays, described in examples 11 to 17, have identified the 105KDa protein as a α V β 3 integrin and a significant receptor activity of integrins comprising subunits other than α V and β 3.

[0077] In particular, experiments reported in examples 13 to 17 showed a significant ability of integrins to act as receptors for flaviviruses wherein subunits have an independent ability to act as a receptor for the virus. In particular, a particularly significant ability of integrin subunits αV and $\beta 3$ to act as a receptor was observed. However a better efficiency is obtained when both subunits are present.

[0078] These results have been obtained in Vero cells and N2A cells. However, since integrins are expressed in most types of cells, including brain cells of vertebrates, and since flaviviruses, and in particular WNV, have been shown to afflict a range of other mammals such as horses and humans, as well as other vertebrates such as birds, the scope of these results can be extended to these other systems as well.

[0079] The administration of an agent able to functionally interfere with the integrin has been shown to affect the flavivirus entry in the host cell (see examples 1-22).

[0080] Therefore, the present disclosure shows that administration of an agent that functionally interferes with integrin affects flavivirus entry in the host cell. In particular, agents able to interfere with the functionality of the attachment domain of the integrin are functionally interfering agents of this disclosure.

[0081] Functionally interfering agents can enhance or inhibit the integrin functionality. In particular, interfering agents able to functionally inhibit integrins, such as functional blocking antibodies and competitive ligands, are considered to be a functionally interfering agent able to inhibit the entry of flavivirus.

[0082] Preferably, the functionally blocking antibodies are polyclonal antibodies, in particular against the 105 KDa protein, the integrin subunits αV , $\beta 3$, $\alpha V\beta 3$ or $\alpha V\beta 5$.

[0083] The competitive ligand can be a natural ligand, such as for example fibronectin, vitronectin or laminin, or a synthetic ligand, for example RGD peptide or chemically synthesized peptides that are complementary to the binding region in the integrin, which ligand can be identified and manufactured by a person skilled in the art, based on the information provided in the present application.

[0084] Proteases such as papain, glycosidases, lectins, and cycloheximide are also considered functionally interfering agents able to inhibit the integrin functionality.

[0085] These functionally interfering agents were used at a range of concentrations that are non-cytotoxic to the cells used in our system. In particular, functionally inhibitors of the integrin can be administered at following amounts: papain 10-50mUnit/ml; Lectin and in particular Concanavalin-A phytohemagglutinin) 100-1000 μ g/ml; cycloheximide about 100 μ g/ml; Endoglycosidase 10-100 mUNIT/ml; O-glycosidase 0.1-1 mUNIT/ml; mannosidase 100-1000 μ g/ml; Fucosidase 10-100 mUNIT/ml.

[0086] With reference to competitive ligands, effective concentrations that will block the entry of West Nile virus will be in the ranges that follows: RGE peptide: 0.01-30 μ g/ml; RGD peptide: 0.01-30 μ g/ml; Fibronectin: 0.01-40 μ g/ml; Vitronectin: 0.01-40 μ g/ml; Laminin: 0.01-40 μ g/ml; Chondroitin sulphate: 0.01-40 μ g/ml; Heparin: 0.01-40 μ g/ml. These ligands can be administered before an infection occurs or during an infection to block the entry of the subsequent newly produced virus progeny from entry. The ligand needs to be incubated with the cells for at least 30 min for effective binding to the cells and can be present for more than 1 hr. The temperature for incubating the ligand with cells can be in the range of about 4°C to 40°C.

[0087] With reference to the antibodies against integrin subunits, the effective concentrations of the functional blocking integrin (all the integrin used) antibodies that will block the entry of West Nile virus will be in the range of about 0.025 μ g/ml – 40 μ g/ml. These antibodies can be administered before an infection occurs or during an infection to block the entry of the subsequent newly produced virus progeny from entry. The antibodies are preferably incubated with the cells for at least 10 min for effective binding to the cells and can be present for more than 1 hr. The temperature for incubating the antibodies with cells can be in the range of about 4°C to 40°C.

[0088] The present disclosure also shows that agents interfering with the expression of the integrin are able to affect the entry of the flavivirus into the host cell.

[0089] In particular, interference with the expression of the integrin may result in inhibition or enhancement of such expression. For example, a silencer or preferably a short interfering RNA is suitable for use with the present invention and allows flavivirus activity to be inhibited. Other agents interfering with the expression of the integrin are

identifiable by a person skilled in the art based on the information provided in the present application.

[0090] The present disclosure shows that entry of flavivirus is also affected by administration of an agent functionally interfering with the functionality of an ATPase in the cell. In particular the ATPases as intended herein include but are not limited to the plasma membrane associated ATPases (known as F-ATPases) and the vacuolar ATPases that can be localized to the plasma membrane as well as the membrane of endocytic vesicles and lysosomes.

[0091] Functionally interfering agents can enhance or inhibit the ATP-ase functionality. In particular, interfering agents able to functionally inhibit the ATPases, such as functional blocking antibodies and competitive ligands, are examples of functionally interfering agents able to inhibit the entry of flavivirus.

[0092] Preferably, the functionally blocking antibodies are monoclonal and polyclonal antibodies in particular against extracellular subunits of ATPases (plasma membrane) as well as V-ATPases. It has been shown that such antibodies are particularly effective in inhibiting flavivirus entry (See Example 19).

[0093] The competitive ligand can be a natural ligand or a synthetic ligand, such as chemically synthesized peptides that are complementary to binding regions in the ATPase, which can be identified and manufactured by a person skilled in the art based on the information provided in the present application.

[0094] Administration of an agent that functionally interferes with a neurotensin receptor affects flavivirus entry in the host cell is disclosed. Neurotensin receptors are present in the brain and gastrointestinal tract and are involved in neurotransmission.

[0095] Functionally interfering agents can enhance or inhibit the neurotensin functionality. In particular, interfering agents able to functionally inhibit a neurotensin receptor, such as functional blocking antibodies and competitive ligands, are examples of functionally interfering agents able to inhibit the entry of flavivirus

[0096] Preferably, the functionally blocking antibodies are monoclonal and polyclonal antibodies, directed, in particular, against the extracellular portion of the neurotensin receptor.

[0097] More specifically, the inventors show that, in particular competitive natural ligands such as neurotensin and antibodies against the receptor, inhibit the entry of the virus (See Examples 20-21). Other competitive ligands known to compete with neurotensin for neurotensin receptor can be used. For example, Neuromedin N, 8 bromo-cAMP, IBMX and forskolin are competitive ligands competing with neurotensin for the neurotensin receptor (Shi & Bunney 1992), which are expected to inhibit the entry of the virus.

[0098] The competitive ligand can also be a synthetic ligand, such as chemically synthesized peptides that are complementary to the binding region in the neurotensin receptor. Also in this case, ligands can be identified and manufactured by a person skilled in the art based on the information provided in the present application.

[0099] The present disclosure provides a further method for interfering with flavivirus entry. According to this further method, domain III in the envelope protein of the flavivirus has been found to bind the flavivirus receptor in the host cell.

[00100] Experiments of competitive binding between the domain III of the virus envelope protein, expressed in soluble form and West Nile and dengue viruses, extensively reported in Example 22, show that the domain III of the envelope protein is the attachment domain of the envelope protein for the receptor protein in the cell.

[00101] Therefore, administration of an agent that functionally interferes with domain III of the envelope protein also affects flavivirus entry in the host cell. The agent functionally interfering with the domain III activity can enhance or inhibit the functionality of domain III. Preferably, the functionally interfering agent is able to inhibit the functionality of domain III. In particular, agents such as a competitive ligand of domain III or an antibody against domain III, are functionally interfering agent able to inhibit the functionality of the domain III.

[00102] In particular, the competitive ligand can be a competitive natural ligand of domain III such as an integrin, and more specifically an integrin comprising at least one of αV and $\beta 3$ integrin subunits, preferably an integrin comprising both subunits αV and $\beta 3$. The competitive natural ligand can be also a neurotensin receptor or an ATPase, preferably an F-ATPase or V-ATPase or a molecule substantially homologous thereto.

The competitive ligand can also be a synthetic ligand, such as chemically synthesized peptides that are complementary to domain III or the binding region in the integrin or neurotensin or ATPase, which can be identified and manufactured by a person skilled in the art based on the information provided in the present application. The antibody against the domain III is preferably a polyclonal antibody against domain III, most preferably a functional blocking polyclonal antibody against domain III.

[00103] An antibody against a membrane 105KDa polypeptide having a sequence substantially homologous to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO: 4 and SEQ ID NO:5 is also here disclosed.

[00104] A kit of parts for controlling and assaying entry of the flavivirus in a cell is also disclosed, comprising the flavivirus and at least one among the functionally interfering agents here described and/or agents interfering with the integrin expression.

[00105] The flavivirus and the at least one among the functionally interfering agents are comprised in the kit independently in one or more compositions wherein each is in a composition together with a suitable vehicle carrier or auxiliary agent.

[00106] In particular, the flavivirus and functionally interfering agents or expression-interfering agents interfering with the expression can be provided in the kits, with suitable instructions and other necessary reagents, in order to perform the methods here disclosed. The kit will normally contain the compositions in separate containers. Instructions, for example written or audio instructions, on paper or electronic support such as tapes or CD-ROMs, for carrying out the assay, will usually be included in the kit. The kit can also contain, depending on the particular method used, other packaged reagents and materials (i.e. wash buffers and the like).

[00107] Further details concerning the identification of the suitable carrier agent or auxiliary agent of the compositions, and generally manufacturing and packaging of the kit, can be identified by the person skilled in the art upon reading of the present disclosure.

[00108] A method for inducing immunity to a flavivirus in a vertebrate susceptible to infection to the flavivirus is also disclosed. In particular, the method is effective for immunizing against infection to a flavivirus having an envelope protein comprising the

domain III and comprises administering an immunogenic effective amount of an amino acidic molecule comprising a domain III of the envelope protein of the flavivirus. Preferably, the domain III comprises a portion having a sequence substantially homologous to SEQ ID NO: 19 or SEQ ID NO: 21.

[00109] Accordingly, also a vaccine for flavivirus infection is disclosed, comprising, as an active agent, a polypeptide comprising domain III of the envelope protein of the flavivirus. When the flavivirus is West Nile Virus, the polypeptide preferably comprise a portion having a sequence substantially homologous to SEQ ID NO: 19 or SEQ ID NO: 21.

[00110] Also a DNA vaccine comprising as an active agent a vector wherein the domain III sequence has been disclosed, in particular an adenovirus replication defective vector is expected to be effective, as well as a chimera peptide vaccine against a flavivirus comprising as an active agent a chemically synthesized peptide of the flavivirus envelope domain III region, in particular when the flavivirus is West Nile Virus or dengue.

[00111] The vaccine may advantageously contain other components, such as adjuvant, attenuated flavivirus, killed flavivirus or subunits thereof, and/or other immunogenic molecules against the flavivirus, in particular against the envelope protein of the flavivirus. The manufacturing process and components to be included in the vaccine are known as such to the person skilled in the art and will not be disclosed here in detail.

[00112] Also a method of treating a flavivirus infection in a vertebrate in need of such a treatment, in particular humans, is disclosed. This method comprises administering to a subject in need of such treatment an immunologically effective dose of the vaccine here disclosed.

[00113] The vaccine disclosed herein may be administered by any suitable route, which delivers an immunoprotective amount of the domain III and other immunogenic components of the vaccine to the subject. Routes of administration of the vaccine, such as, for example, parenteral route, intramuscular route or deep subcutaneous route, are identifiable by a person skilled in the art. Other modes of administration may also be

employed, where desired, such as oral administration or via other parenteral routes, i.e., intradermally, intranasally, or intravenously.

[00114] A person skilled in the art can determine the appropriate immunoprotective and non-toxic dose of such vaccine to be administered. The appropriate immunoprotective and non-toxic amount of the active agents in the vaccine are in the range of the effective amounts of antigen in conventional vaccines including active agents. The specific dose level for a specific patient will be determined with reference to the age, sex, and general health of the patient. Also, the synergistic effect with other drugs administered as well as the diet of the patient, the time and route of administration, and the degree of protection to be sought, will be taken in consideration to determine the appropriate immunoprotective dose for the patient. The administration can be repeated at suitable intervals, if necessary.

[00115] A method for treating a flavivirus infection in a vertebrate, when the flavivirus exhibits a flavivirus envelope protein comprising a domain III is also disclosed. A pharmaceutically effective amount of an agent functionally interfering with the domain III of the envelope protein of the flavivirus is administered.

[00116] Also a pharmaceutical composition is disclosed herein, for treatment of a flavivirus infection in a vertebrate when the flavivirus exhibits an envelope protein comprising domain III. The pharmaceutical composition disclosed comprises a pharmaceutically effective amount of an agent interfering with domain III of the envelope protein and a pharmaceutically acceptable carrier, vehicle or auxiliary agent.

[00117] The agent functionally interfering with domain III of the flavivirus in the methods of treatment and pharmaceutical composition can be any agent functionally interfering with domain III, able to inhibit the entry of the flavivirus in the cell herein disclosed. Preferably, the agent is a functional blocking antibody against domain III, most preferably a functional blocking polyclonal antibody against domain III, in particular murine antibodies. Also preferred is a competitive ligand of domain III, which can be a competitive natural ligand such as an integrin including at least one of subunits αV or $\beta 3$, most preferably the integrin $\alpha V\beta 3$, a neurotensin receptor or an ATPase, preferably a F-ATPase or a V-ATPase. Preferred agents functionally interfering with domain III,

able to inhibit virus entry also include competitive synthetic ligands, such as chemically synthesized peptides that are complementary to domain III or the binding region on the integrin α V β 3, neurotensin receptor and/or F-ATPase or V-ATPase.

[00118] A pharmaceutically acceptable carrier, vehicle or auxiliary agent as used herein can be identified by a person skilled in the art as suited to the particular agent and to the particular dosage form desired. The composition may be prepared in various forms for administration, identifiable by a person skilled in the art.

[00119] The agent functionally interfering with domain III as described above may be administered using any amount and any route of administration effective for attenuating infectivity of the virus. Thus, the expression "pharmaceutically effective amount", as used herein, refers to a nontoxic but sufficient amount of the antiviral agent to provide the desired treatment of viral infection.

[00120] The agent described herein may be administered as such, or in the form of a precursor from which the active agent can be derived. Such a precursor is a derivative of a compound described herein, the pharmacologic action of which results from the conversion by chemical or metabolic processes in vivo to the active compound. Such a precursor may be prepared according to procedures well known in the field of medicinal chemistry and pharmaceutical formulation science for each agent described herein.

[00121] The administration of the agent functionally interfering with domain III may be performed by routes identifiable by the person skilled in the art depending on the agent administered and the nature and severity of the infection to be treated.

[00122] The exact amount required for the treatment of a subject, and route of administration of such amount will vary from subject to subject, depending on the species, age, and general condition of the individual patient, the severity of the infection, the particular antiviral agent and its mode of administration, etc.

[00123] In view of the inhibitory effect on flavivirus infection, the agent interfering with domain III of the flavivirus envelope protein will be useful not only for therapeutic treatment of virus infection, but also for virus infection prophylaxis.

[00124] A method for diagnosing a flavivirus infection in a vertebrate susceptible to infection by the flavivirus is also disclosed. The method comprises: contacting a sample

tissue from the vertebrate with an agent able to bind domain III of the envelope protein of the virus, in particular an antibody against domain III, a ligand of domain III or a molecule substantially homologous thereto, associated with an identifier; and detecting presence or absence of a flavivirus-integrin complex or flavivirus-neurotensin complex by detecting presence of the identifier. Alternatively, the identifier can be associated with the sample tissue. Preferably, the antibody against domain III are polyclonal antibodies, and the ligand is a competitive natural ligand such as integrin, a neurotensin receptor protein or an ATPase or a competitive synthetic ligand.

[00125] For example, a plasma membrane of the sample tissue or cell line can be extracted, for example with the protocol given in the reference (Chu and Ng, 2003). The extracted plasma membrane can be coated onto 96 well plates and Cy5-labelled WNV particles can be added for the interaction. After extensive washing to remove background noise, the presence of WNV particles binding to the receptor molecules can be detected, for example by fluorescence in an ELISA plate reader.

[00126] A kit for the diagnosis of a flavivirus infection comprising at least one of the above agents able to bind domain III, optionally associated or to be associated with an identifier, and one or more reagents able to detect the identifier, is also disclosed, wherein the agent able to bind domain III and the reagents are used according to the diagnostic method herein disclosed.

[00127] The agent able to bind domain III and the one or more reagents able to detect the identifier, can be independently included in one or more compositions wherein they are comprised together with a suitable vehicle carrier or auxiliary agent. The identifier can also be included in such compositions or in a separate composition to be associated with the agent able to bind the domain or with the cell or sample to be tested.

[00128] The identifier and the reagent able to detect the identifier, are identifiable by a person skilled in the art. Other compositions and/or components that may be suitably included in the kit and are also identifiable by a person skilled in the art.

[00129] Also a diagnostic method to detect whether a sample tissue or cell line is susceptible to flavivirus infection is disclosed, comprising contacting a cell with an

identifier for the presence or expression of an integrin, neurotensin receptor and or ATPase to be associated with the presence or expression of an integrin, neurotensin receptor and or ATPase, and detecting the presence of the identifier associated to presence or expression of an integrin, neurotensin receptor and/or ATP-ase in the cell.

[00130] For example, an approach to detect whether a sample tissue or cell line is susceptible to flavivirus infection can be that of detecting the presence or expression of integrin alphaV beta 3 or neurotensin by staining these cells with antibodies against these receptors and detect for fluorescence. An exemplary alternative can be to use real-time quantitative PCR to detect the presence of mRNA for integrin or neurotensin receptor in the tissue sample or cells.

[00131] A diagnostic kit to detect whether a sample tissue or cell line is susceptible to flavivirus infection is also disclosed, comprising an identifier for the presence or expression of an integrin, neurotensin receptor and or ATP-ase, and a reagent able to detect the presence of the identifier. The identifier and the reagent able to detect the presence of the identifier are to be used in the method to detect whether a sample tissue or cell line is susceptible to flavivirus infection here disclosed.

[00132] The identifier and the reagent can be included in one or more compositions where the identifier and/or the reagent are included with a suitable vehicle, carrier or auxiliary agent.

[00133] In both of the diagnostic kits herein disclosed, the agents and identifier reagents can be provided in the kits, with suitable instructions and other necessary reagents, in order to perform the methods here disclosed. The kit will normally contain the compositions in separate containers. Instructions, for example written or audio instructions, on paper or electronic support such as tapes or CD-ROMs, for carrying out the assay, will usually be included in the kit. The kit can also contain, depending on the particular method used, other packaged reagents and materials (i.e. wash buffers and the like).

[00134] Further details concerning the identification of the suitable carrier agent or auxiliary agent of the compositions, and generally manufacturing and packaging of the

kit, can be identified by the person skilled in the art upon reading of the present disclosure.

[00135] Methods, kits, vaccine and pharmaceutical compositions disclosed herein are particularly used when the flavivirus is a member of the Japanese encephalitis serocomplex, preferably West Nile Virus, Japanese Encephalitis virus, West Valley or a virus such as Dengue and Kunjin virus. Preferably, the vertebrate is a mammal, and, in particular, a human being.

[00136] A person skilled in the art can identify modalities, dosages, timing of administration of the methods herein disclosed as well as vehicle carrier auxiliary agents, relative concentration, formulation and modalities of administration of the compositions herein disclosed.

[00137] As used herein, the term "antibody" may be a polyclonal or monoclonal antibody unless differently specified. The relevant preparation, is identifiable by a person skilled in the art upon reading of the present disclosure. In the specific examples given, murine polyclonal antibodies were used. Monoclonal antibodies may be obtained by any technique that provides for the production of antibody molecules by continuous cell line culture. These techniques are well known and routinely used in academic and industrial settings. Some techniques include but are not limited to the hybridoma technique of Kohler and Milstein, (1975, *Nature* 256:495-497; and US Patent Number 4 376 110), the human B-cell hybridoma technique (Kosbor et al., 1983, *Immunology Today* 4:72; Cole et al., 1983, *Proc. Natl Acad. Sci. USA* 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, *Monoclonal Antibodies And Cancer Therapy*, Alen R. Liss, Inc., pp. 77-96).

[00138] Antibody fragments, which retain the ability to recognize the antigen of interest, are included as well.

[00139] The antibodies are produced using techniques known to those skilled in the art and disclosed, for example, in immunization techniques *in vivo* or *in vitro*. These techniques are well known and routinely used in academic and industrial settings.

[00140] As used herein, the terms "polypeptide", and "protein" refer to a polymer of amino acid residues with no limitation concerning a minimum length of the product. The

definition encompasses peptides, oligopeptides, dimers, multimers, and the like, full-length proteins and fragments thereof. The terms also include polypeptides subjected to post-expression modifications such as, for example, glycosylation, acetylation, phosphorylation and the like. Additionally, the term "polypeptide" refers also to a modified protein including protein comprising deliberate or accidental modifications of the original sequence, such as deletions, additions and substitutions, so long as the protein maintains the desired activity.

[00141] As used herein, the term "homology" refers to the percent similarity between two polynucleotide or two polypeptide moieties. Two DNA, or two polypeptide, sequences, are "substantially homologous" to each other when the sequences exhibit at least about 50% , preferably at least about 70%, more preferably at least about 80%-85%, preferably at least about 90%, and most preferably at least about 95%-98% sequence similarity over a defined length of the molecules. As used herein, substantially homologous also refers to sequences showing complete identity to the specified DNA or polypeptide sequence.

[00142] As used herein the term "agent functionally interfering" refers to any kind of interference with the functionality of the molecule, which results in a different functionality of the molecule compared with the functionality registered in absence of the agent. This includes enhancing and inhibiting the functionality of the molecule. Examples of interference with the functionality of the molecule may be obtained include but are not limited to binding the molecule, interference by steric hindrance with the molecule, modify functional components of the molecule, interfere with the expression of the molecule.

[00143] The following examples are provided to describe the invention in further detail. These examples, which set forth a preferred mode presently contemplated for carrying out the invention, are intended to illustrate and not to limit the invention.

Examples

[00144] General materials and methods used throughout the experiments will first be presented.

Maintenance of cell lines

[00145] Vero cells (Green monkey kidney) were grown in Medium 199 (M199) containing 10 % inactivated fetal calf serum (FCS). Murine neuroblastoma (N2A) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % FCS. Polarized Vero C1008 cells were grown on 0.4 μ m porous support membrane insert immersed in M199 supplemented with 10 % FCS. The polarity of the cell monolayers was monitored by measuring the transepithelial electrical resistance with millicell-ERS apparatus. The net resistance of the confluent cell monolayers was maintained at 50-70 $\Omega\cdot\text{cm}^2$ as calculated based on Blau & Compans (1995). Neurons are isolated from mice and maintained in Dulbecco's modified Eagle's medium supplemented with 10 % FCS.

Virus growth & purification

[00146] Vero cells were used to propagate a Flavivirus, West Nile (Sarafend) virus throughout this study. Confluent monolayers of Vero cells were infected with West Nile virus at a multiplicity of infection (MOI) of 10 PFU/ml. At 24 hr post infection (p.i.), the supernatant was harvested by centrifugation at 5000 rpm for 10 minutes. West Nile viruses were then concentrated and partially purified by centrifugal filter device at 2000 rpm for 2 hours. The partially purified viruses were then applied to a 5 ml 25% sucrose cushion for further purification. Sucrose gradient was centrifuged at 25,000 rpm for 2.5 hours at 4 °C in a SW55 rotor. Finally, the purified virus pellet was re-suspended in TNE buffer (50 mM Tris-HCl, 100 mM NaCl, 1mM EDTA). The re-suspended virus was aliquoted, snapped frozen and stored at -80°C. The titer of the purified virus preparation was determined by plaque assay on Vero cells and was found to be 9×10^8 – 5×10^9 PFU/ml. As a control, supernatant of uninfected Vero cells were processed as described above. A similar method was used to propagate Kunjin virus cells, a closely related virus in the same subgroup, and Dengue virus cells, another flavivirus.

Antibodies and reagents

[00147] The antibody for West Nile virus envelope (E) protein was a monospecific polyclonal antibody raised in rabbit. The secondary antibody conjugated to Texas Red (TR) was purchased. Fifteen-nanometer Protein-A colloidal gold was also purchased for antibody detection.

[00148] Polyclonal antibodies against the 105 kDa and WNV envelope DIII protein have been produced according to the following. The 105-kDa plasma membrane protein (from Vero cells) or WNV envelope DIII protein was excised from the SDS-10% PAGE gels, homogenized, and incubated with ImmunEasy mouse adjuvant (Qiagen, USA) at a concentration 3 µg of protein per 30 µl of adjuvant. The antigen-adjuvant mixture was used to immunize BALB/c mice five times subcutaneously at 14-day intervals. Mouse sera were collected 12 days after the last booster. Mouse sera were purified using Econo-Pac serum IgG purification kits (Bio-Rad, USA) and dialyzed overnight with PBS. The purified immunoglobulins were stored at -20°C. Sera were tested by Western blotting detection for the presence and specificity of antibody against the 105-kDa membrane protein or DIII protein.

Cell membrane protein preparation

[00149] The plasma membrane proteins are prepared as described by Martinez-Barrage and del Angel (2001) and Salas-Benito and del Angel (1997). The integrity of extracted membrane proteins was determined by electron microscopy as described by Atkinson and Summers (1971). The concentration of the protein was determined by Bradford assay with bovine serum albumin (BSA) as the standard. Approximately 800 µg of proteins were obtained. The membrane protein preparation was aliquoted and stored at -20 °C.

Example 1: Protease, phospholipase, glycosidase and lectin treatment of cells.

[00150] To determine the biochemical components (e.g. lipids, proteins or carbohydrates) of West Nile virus receptor molecules on the surface of Vero cells, cells were pretreated with a panel of enzymes or chemicals that would destroy the individual membrane components.

[00151] Cell monolayers (Vero cells or N2A) of approximately 5×10^6 cells were washed twice with phosphate buffer saline (PBS) before enzyme treatment. Cell monolayers were incubated with the proteases and phospholipases, glycosidases and lectins (as listed below) in PBS at a pH of 7.0 for 45 min at 25°C. After treatment, cell monolayers were washed twice with PBS supplemented with 2% FCS to remove the enzymes. Cell monolayers were then incubated with West Nile virus (MOI = 10) at 37 °C

for 1 hour. Excess virus particles were inactivated with sodium citrate buffer (pH 2.8) for 10 minutes and the cell monolayer washed twice with PBS. Vero cells were then incubated at 37°C for 12 hours. At 12 hours p.i., virus titers from the treated samples were determined by plaque assays. Three independent experiments were conducted for each set of enzymes used.

[00152] The enzymes used were; Proteases: Proteinase K (EC 3.4.21.64) from *Tritirachium album*, concentration of 10 µg/ml, 1 µg/ml & 0.1µg/ml; α -chymotrypsin (EC 3.4.21.1) from bovine pancreas, concentration of 10 µg/ml & 1 µg/ml; trypsin (EC 3.4.21.4) from bovine pancreas, concentration of 10 µg/ml, 1 µg/ml & 0.1 µg/ml; Bromelain (EC 3.4.22.32) from pineapple stem, concentration of 20 mUnit/ml, 2 mUnit/ml & 0.2 mUnit/ml; Papain (EC 3.4.22.2) from *Carica papaya*, concentration of 50 mUnit/ml & 10 mUnit/ml. Phospholipases: Phospholipase A₂ (EC 3.1.1.4) from bovine pancreas, concentration of 1 UNIT/ml & 0.1 UNIT/ml; Phospholipase C (EC 3.1.1.4.3) from *Clostridium perfringens*, concentration of 10 UNIT/ml & 1 UNIT/ml; Phospholipase D (EC 3.1.4.4) from peanut, concentration of 100 UNIT/ml & 10 UNIT/ml; Glycosidases: Endoglycosidase H (EC 3.2.1.96) from *Streptomyces plicatus*, concentration of 100 mUNIT/ml & 10 mUNIT/ml; O-glycosidase (EC3.2.1.97) from *Diplococcus pneumoniae*, concentration of 1 mUNIT/ml & 0.1 mUNIT/ml; α -mannosidase (EC 3.2.1.24) from almonds, concentration of 1000 µg/ml, 100 µg/ml & 10 µg/ml; α -Fucosidase (EC 3.2.1.11) from almond meal, concentration of 100 mUNIT/ml & 10 mUNIT/ml; Heparinase I (EC 4.2.2.7) and Heparinase III (EC 4.2.2.2.8) from *Flavobacterium heparinum*, concentration of 1 UNIT/ml & 0.1 UNIT/ml;. Lectins : Concanavalin-A from Jack bean, wheat germ agglutinin from *Triticum vulgaris*, phytohemagglutinin from *Phaseolus spp.*, concentration of 1000 µg/ml & 100 µg/ml ; Sodium periodate concentration of 1 mM & 0.1 mM. Cell viability after enzyme treatment was assessed by Trypan blue staining and observation under phase contrast microscope BX 60.

[00153] Treatment with glycosidases, sodium periodate and lectins was made to investigate possible involvement of carbohydrate moieties on the plasma membrane for West Nile Virus. In particular, lectins (highly specific carbohydrate binding molecules) are widely used to determine the nature of carbohydrates involved in ligand-receptor

interaction (Liener *et al.*, 1986). Vero cells were then incubated with lectins such as Wheat germ agglutinin (which binds to GlcNac β 1-4 on N-linked glycans), concanavalin A (which binds to α -linked terminal mannose residues on N-linked glycans) and phytohemagglutinin (which binds oligosaccharides) to assess their effects on West Nile virus entry.

[00154] The enzymes or chemicals were used at concentrations known to be effective in reducing the entry of other known viruses (Borrow and Oldstone, 1992; Ramos-Castaneda *et al.*, 1997; Salas-Benito and del Angel, 1997; Martinez-Barragan and del Angel, 2001). Results are expressed as the number of log unit inhibition with respect to untreated samples. At the same time, cell viability after treatments was also assessed by Trypan blue exclusion method. The number of viable cells after treatments was not significantly different from untreated (control) numbers.

[00155] Figures 1A and 1B show the effects of phospholipases, proteases, glycosidases, sodium periodate and lectins treatments on Vero cells and the subsequent ability of the cells to allow West Nile virus infection.

[00156] In particular, results shown in Figure 1A, demonstrate that treatment of Vero cells with the three phospholipases does not cause any significant reduction in the productive yields of West Nile virus. Vero cells were also treated with a panel of proteases which included both serine and thiol proteases. Pretreatment of Vero cells with proteases exhibited a dosage-dependent inhibition of West Nile virus entry. Papain, a cysteine endopeptidase that solubilized integral membrane protein, showed the highest inhibition (approximately a 5-log unit inhibition) of West Nile virus infection. Therefore, these results show that the cellular receptor molecule responsible for West Nile virus entry is of a proteineous nature.

[00157] In particular, results shown in Figure 1B demonstrate the effects of treatment with heparinases, glycosidases and sodium periodate. With reference to heparinases, pretreatment of a cell with heparinases has no effect on the entry of West Nile virus. This result was further supported by a virus entry blockage study using anti-heparan sulfate proteoglycan treatments of cells.

[00158] As per the glycosidases, both Endoglycosidase H and α -mannosidase (which hydrolyzes N-linked oligosaccharides with mannose structures and α -mannose residues respectively) had a significant inhibition on West Nile virus binding and entry into Vero cells. In agreement with this result, pretreatment of cells with sodium periodate also substantially reduced the binding ability of the cells for West Nile virus. Sodium periodate works by oxidizing cell surface carbohydrate residues, but without altering protein or lipid epitopes. As for O-glycosidase, α -fucosidase and Heparinase I and III treatments, these enzymes had minimal effect on West Nile virus infection. In this series of glycosidase treatments, protease inhibitors cocktails were included to prevent possible contamination by proteases.

[00159] With reference to treatment with lectins, blocking of the mannose residues on N-linked glycans with concanavalin-A on the cell surfaces prevented the entry of West Nile virus into Vero cells.

[00160] Therefore, these preliminary results suggested that the West Nile virus cellular receptor molecule(s) on Vero and N2A cells is a glycoprotein with complex N-linked sugars containing α -mannose residues.

Example 2: Isolation of receptor protein

[00161] In these experiments, plasma membrane proteins were isolated and purity was checked under the electron microscope. The purity of the plasma membrane extract was considered acceptable with reference to Atkinson and Summers (1971). Equal quantities of the membrane proteins were loaded into different gel lanes and separated by SDS-PAGE and transferred onto nitrocellulose membranes. The nitrocellulose membranes were incubated sequentially with purified West Nile virus, rabbit polyclonal mono-specific antibody against the viral envelope protein and detection by secondary anti-rabbit antibodies conjugated with alkaline phosphatase with addition of substrate (NBT).

[00162] In particular, in order to isolate the West Nile virus binding cell receptor proteins in Vero and N2A plasma membrane extracts, VOPBAs were performed. Membrane proteins (80 μ g) from either Vero or N2A cells were separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) as described by

Sambrook and co-workers (1989). Proteins separated by SDS-PAGE were electrophoretically transferred onto a nitrocellulose membrane using a Western Blotting transfer apparatus for 3 hours at 4 °C. The nitrocellulose membrane was soaked overnight in a milk buffer (5% skim milk and 0.5% BSA) to block non-specific binding sites and to allow re-naturation of the separated proteins. The membrane was rinsed with PBS (three times) and incubated sequentially with (a) the purified West Nile virus (prepared as describe above) for 6 hours at 4 °C (b) mono-specific polyclonal antibodies against West Nile virus envelope protein for 1 hour at 37 °C (c) secondary antibody (anti-rabbit IgG) conjugated with alkaline phosphatase (Chemicon Int, USA) for 45 minutes at 37 °C. All incubations were carried out on a rocking platform and membranes were washed three times with a washing buffer (containing 50 mM Tris and 200 mM NaCl with 0.05% Tween 20). Non-specific binding of the virus particles was reduced with a high salt buffer wash. The presence of virus binding was detected by the addition of substrate, nitroblue tetrazolium. Finally, the membranes were washed with distilled water and dried (and/or put in stripping and re-probing protocol).

[00163] A 105-KDa band was detected. The corresponding 105-kDa plasma membrane protein (from Vero cells) was excised from the SDS-10% PAGE gels, homogenized and incubated with ImmunEasy mouse adjuvant at a concentration recommended by the manufacturer, Qiagen. The antigen-adjuvant mixture was used to immunize BALB/C mice five times subcutaneously at 14-day intervals. Mouse sera were collected 12 days after the last booster. Mouse sera were purified using Econo-Pac serum IgG purification kits and dialyzed overnight with PBS. The purified immunoglobulins were stored at –20 °C. Sera were tested by Western blotting detection for the presence and specificity of antibody against the 105-kDa cell receptor protein.

[00164] Results are shown in Figures 2A and 2B. West Nile virus was observed to bind to a 105-kDa band in the membrane preparations of both Vero and N2A cells (Figures 2A and 2B, respectively, Lane 1). No bands were observed when supernatants of uninfected cells (prepared as according to the supernatants of West Nile virus-infected cells) were incubated under the same conditions (Figures 2A and 2B, respectively, Lane 2).

[00165] To ensure this was a specific interaction between the virus and the 105-kDa cell receptor protein, several procedures were carried out. In particular, despite high salt (200 mM NaCl) and detergent (0.05% Tween 20) washing, West Nile virus still binds strongly to the 105-kDa cell receptor proteins.

Example 3: Protease treatment of membrane proteins

[00166] To affirm the results of enzymes and chemical treatments of intact Vero cells reported in the previous examples, VOPBAs were also performed on plasma membrane protein extracts that were treated with protease (papain).

[00167] After papain treatment of both vero and N2A cells, the membrane proteins were isolated and prepared and VOPBAs were performed according to the procedure described in example 2.

[00168] The results are shown in Figure 3. WN virus binding to the membrane proteins of both Vero and N2A cells after papain treatment at the concentration of 50 mUnit/ml is abolished (Lanes 2 & 4 respectively) as compared to that of the untreated cells (Lanes 1 & 3 respectively). These results confirm that the receptor is of a proteinaceous nature. Additionally, since also after this treatment a 105 KDa band was observed, the existence of a 105-KDa virus receptor was further confirmed.

Example 4: Kinetics of West Nile virus binding molecules

[00169] Following the experiments reported in example 3, the kinetics of West Nile virus binding molecules returning to the cell surface after removal with papain was also examined.

[00170] Vero cells (approximately 5×10^6 cells) were treated with papain at 50 mUnit/ml in PBS or PBS (untreated) for 45 minutes at 25 °C. Another set of Vero cells was incubated with 100 µg/ml of cycloheximide to block new protein synthesis, for 2 hours prior to papain treatments. Treated cells were washed twice with PBS supplemented with 2% FCS to inactivate the enzyme. Fresh M199 plus 10% FCS with or without cycloheximide (100µg/ml) were added to the cells. The cells were then incubated at 37 °C in 5% CO₂. At specific times after incubation (0, 2 & 4 hours), plasma membrane proteins were extracted as herein described and VOPBA was performed.

[00171] The results of these experiments are shown in Figure 4. Vero cells were first treated with papain (50 mU/ml) for 45 minutes at room temperature and after protease (papain) removal after 0, 2 and 4 hours, virus binding was determined by VOPBA. No virus binding was observed at 0 hr following the removal of the protease [Figure 4 – Lane 2 (without cycloheximide and Lane 5 (with cycloheximide)) when compared to the membrane protein that is not treated with papain (Lane 1). West Nile virus binding to the 105-kDa cell receptor protein was observed after 2 hours with the removal of the protease and reached its original level within 4 hours (Figure 4 – Lanes 3 & 4 respectively). Despite the blockage of new protein synthesis by cycloheximide, virus binding was also observed after 2 hours and 4 hours (Figure 4 – Lanes 6 & 7 respectively). This indicates the presence of abundant pre-existing internal pools of the 105-kDa cell receptor proteins that were rapidly trafficked to the cell surface after removal without the need for new protein synthesis to occur.

Example 5: Glycosidase periodate and lectins treatment of membrane proteins

[00172] Carbohydrate residues on cell surfaces have been shown to be important for the initial binding of viruses, which would then mediate the subsequent entry of the virus through its high affinity receptor. The nature and roles of carbohydrate residues present on the 105-kDa protein for West Nile virus binding were further assessed by VOPBA.

[00173] In particular, VOPBAs were also performed on plasma membrane protein extracts (obtained with the procedure reported in example 4) that were treated with glycosidases (Endoglycosidase H, α -mannosidase and O-glycosidase), sodium periodate and lectins.

[00174] After each treatment, membrane proteins were isolated and prepared, and VOPBAs performed according to the procedures described in example 2.

[00175] Results of these experiments are shown in Figures 5A to 5C. Figure 5A shows the results of glycosidase treatments of membrane proteins. No binding of West Nile virus was observed after treatment with α -mannosidase and Endoglycosidase H in both Vero (Lanes 2 and 3) and N2A (Lanes 6 and 7) plasma membrane protein extracts, when compared with the untreated membrane proteins (Vero cells – Lane 1 and N2A cells – Lane 5). In contrast, O-glycosidase treatment of the membrane proteins (Vero

cells – Lane 4 and N2A cells – Lane 8) did not affect the binding of West Nile virus to the 105-kDa protein band. It could be deduced that virus binding to the 105-kDa cell receptor protein is neither mediated by O-linked sugars nor contains O-linked glycosylation. The nature of carbohydrates present in the 105-kDa cell receptor proteins necessary for West Nile virus binding was further assessed by lectin treatments using VOPBA.

[00176] Based on the results shown in Figure 5B, concanavalin-A was observed to block the binding of West Nile virus to the 105-kDa cell receptor proteins in a dosage-dependent manner [Lane 1 (untreated), Lane 2 (10 µg/ml) and Lane 3 (100µg/ml)]. On the other hand, phytohemagglutinin had no effect in blocking virus binding to the cell receptor proteins [equal intensities - Lane 4 (untreated), Lane 5 (10 µg/ml) and Lane 6 100µg/ml)].

[00177] Similarly, Figure 5C shows that binding of West Nile virus to the sodium periodate-treated membrane proteins was reduced in a dosage-dependent manner (Figure 5C, Lane 1 – untreated, Lane 2 – 0.1 mM, Lane 3 – 1 mM and Lane 4 – 10 mM). These results have provided more evidence that the 105-kDa cell receptor protein contains carbohydrate groups with high mannose residues that are important for virus binding.

[00178] Therefore, treatment of membrane proteins with Endoglycosidase H or α -mannosidase abolished virus binding (Figure 5A) while sodium periodate exhibited a dosage dependent inhibition of West Nile virus binding to the 105-kDa cell receptor protein (Figure 5C). Since Endoglycosidase H cleaves only the high mannose residues of N-linked oligosaccharides on glycoproteins and concanavanlin-A binds specifically to mannose residues (Figure 5B), this further emphasizes importance of N-linked sugars with mannose residues on the 105-kDa cell receptor protein for West Nile virus binding.

Example 6: β -mercaptoethanol treatment of membrane proteins

[00179] To investigate the possible presence of di-sulfide-linked bridges in the 105-KDa plasma protein, plasma membrane extracts according to the procedure reported in example 4, were also treated with 5mM of β -mercaptoethanol.

[00180] Interestingly, a faint 105-kDa band and a series of protein bands ranging from 30 to 40-kDa were observed after treatment with β -mercaptoethanol, followed by VOPBA [Figure 6 – Lanes 2 (Vero cells) & 4 (N2A cells)]. Lanes 1 and 3 are untreated samples from Vero and N2A cells, respectively. These results may suggest that treatment with β -mercaptoethanol did not disrupt virus binding and virus binding occurs mainly through the interaction with the carbohydrate moieties instead of the peptide portion of the glycoprotein.

[00181] Consistent with treatment of the membrane proteins with β -mercaptoethanol, West Nile virus was observed to bind to a series of protein bands ranging from 30 to 40-kDa (Figure 6). This may indicate that the 105-kDa cell receptor protein is made up of di-sulfide linked subunits. However, it might be equally plausible that the virus binding protein is actually 30 kDa, and upon cell lysis it becomes cross-linked *via* inadvertent di-sulfide linkage to other proteins.

[00182] To investigate the actual molecular weight of the virus binding protein, the plasma membrane extraction procedure was repeated in the presence of alkylating agent (iodoacetamide) to block thiol reactivities. Consistent with the result obtained in the absence of alkylating agent, West Nile virus binds to a single 105-kDa protein.

[00183] Furthermore, the action of β -mercaptoethanol did not seem to affect virus binding to the protein subunits. This interesting result suggests either that West Nile virus binding did not require a folding dependent di-sulfide bridge or that the West Nile virus binds to the carbohydrates residues on the protein not affected by the action of β -mercaptoethanol. The latter is in line with the above observations that the carbohydrate residues on the membrane protein are necessary for virus binding.

Example 7: Western blot analysis

[00184] For analysis of the specificity of the anti-105-kDa polyclonal antibodies, plasma membrane proteins from Vero cells were separated using SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Biorad, USA). The Western blot procedure was carried out as described in Chu and Ng (2002). The blot was then incubated overnight in the anti-105-kDa protein antibodies or pre-immune serum at room temperature on an orbital shaker for 1 hour. Reactions were then

detected by staining with alkaline-phosphatase conjugated goat anti-mouse IgG (Chemicon Int, USA) with the addition of substrate, nitroblue tetrazolium (NBT).

[00185] Results are shown in Figure 7. The murine polyclonal antibody generated against the 105-kDa protein was shown to be specific for the 105-kDa protein (from Vero cells) by Western blot assay (Figure 7, Lane 2) while no bands were observed after incubation with preimmune serum (Figure 7, Lane 1). This murine polyclonal antibody was also specific for the 105-kDa cell receptor protein from N2A cells.

Example 8: Indirect Immunofluorescence Confocal Microscopy

[00186] To determine the localization of the 105-kDa proteins on Vero cells, immunofluorescence assays were carried out. To determine whether the 105-kDa cell receptor protein is differentially expressed in polarized cells, immunofluorescence assays coupled with optical sectioning by laser scanning confocal microscopy were also carried out with Vero C1008 cells. The Vero C1008 is a polarized cell line derived from Vero cells. These epithelial cells have distinct apical and basolateral domains of the plasma membrane.

[00187] For immunofluorescence microscopy, cell monolayers were grown on cover slips or 0.4 μ m porous support membrane inserts. The subsequent procedure is similar to that described in Chu & Ng (2002). The primary antibody used was anti-105-kDa polyclonal antibody (with a 1:100) and the fluorochrome were Texas Red (TR)-conjugated secondary antibodies. The specimens were viewed with laser scanning confocal inverted microscope (excitation wavelength of 543 nm for TR) using oil immersion objectives.

[00188] Results are shown in Figure 8A. The 105-kDa cell receptor protein was mainly localized to the plasma membrane as detected by the anti-105-kDa protein antibodies using indirect immunofluorescence. This is a typical localization pattern for cell surface molecules. Despite methanol fixation and permeabilization, the murine polyclonal antibodies against the 105-kDa protein still bind specifically to the plasma membrane (arrows).

[00189] Results of experiments performed in polarized cells are shown in Figure 8B. In polarized epithelial cells (Vero C1008), there was a high level of expression of the 105-

kDa proteins at the apical surface as compared to the basolateral surface (Figure 8B). This result could explain for the preferential entry of West Nile virus through the apical surface of the polarized Vero C1008 cells as illustrated in a previous study (Chu and Ng, 2002).

Example 9: Cryo-Immunolabelling Electron Microscopy

[00190] To confirm the results of example 8, cryo-immuno-labelling electron microscopy also was carried out.

[00191] Vero cells were incubated with West Nile virus (MOI = 100) at 4°C for 30 minutes to allow virus attachment to the plasma membrane. The cells were then processed for cryo-electron microscopy using the Tokuyasu method (1984) with some modifications as described in Ng and colleagues (2001). Briefly, the cells were fixed in 4 % paraformaldehyde and 0.2 % glutaldehyde followed by embedding in gelatin. The gelatin block with the cells was immersed in cryo-protectant, rapidly frozen before cryo-ultramicroscopy, using an ultramicrotome (UCT) having a cryo-attachment.

[00192] For immuno-labeling, the primary antibody was the anti-105-kDa membrane protein (1:100 dilution) followed by conjugation with Protein A colloidal gold (at dilution 1:20). The sections were viewed under the CM120 Biotwin transmission electron microscope.

[00193] Results are shown in Figure 9. In particular, localization of the 105-kDa protein was confirmed by the immuno-gold labeling of cryo-sections. More particularly, at the ultrastructural level, West Nile virus was observed to bind to the 105-kDa cell receptor proteins as defined by the 10 nm gold particles – (arrows) at the plasma membrane. The use of immuno-cryo electron microscopy revealed the specific binding of West Nile virus to the 105-kDa cell receptor protein.

Example 10: Inhibition of binding of West Nile virus to membrane cells by receptor protein polyclonal antibodies

[00194] Inhibition of binding of West Nile virus to membrane cells by 105-KDa protein was tested.

[00195] After pre-incubation with the 105 KDa protein antibodies, membrane cells proteins were isolated and prepared, and VOPBAs performed according to the procedure reported in example 2.

[00196] Results are shown in Figure 10. The pre-incubation of the 105 kDa protein antibodies on the separated membrane proteins through VOPBA also prevented the binding of West Nile virus (Figure 10, Lane 1). Virus binding occurred in the absence of the 105-kDa protein antibodies (Lane 2). Hence, these results provide strong evidence that the 105-kDa cell receptor protein is a possible cellular receptor for West Nile virus and other closely-related flaviviruses.

Example 11: Inhibition of West Nile virus infection by receptor protein polyclonal antibodies

[00197] This set of experiments was carried out to determine if the antibodies against the 105-kDa protein recognized the same cell receptor protein for West Nile virus entry. Blockage of West Nile virus cellular receptors with specific antibodies against the cell receptor protein would prevent virus entry.

[00198] Confluent monolayer of Vero cells were first washed twice with PBS and preincubated with preimmune serum or the anti-105-kDa polyclonal antibodies for 1 hour at 37 °C. After incubation, cells were washed thrice with PBS and infected with West Nile virus, Kunjin virus (a flavivirus in the same subgroup as West Nile virus), Dengue (another flavivirus) (MOI = 10). At appropriate p.i. time periods, supernatants from the virus-infected cells were processed for plaque assays. For control purposes, the above procedures were repeated with an unrelated poliovirus infection.

[00199] Results are shown in Table 1 below.

Table 1

		Log unit inhibition of infectivity by pre-immune sera at the following dilutions.		
		1:10	1:100	1:1000
WN Kunjin Polio	0.25 ± 0.56	0.15 ± 0.85	0.20 ± 0.50	
	0.35 ± 0.22	0.28 ± 0.60	0.32 ± 0.68	
	0.95 ± 0.25	0.89 ± 0.87	0.88 ± 0.75	
		Log unit inhibition of infectivity by anti-105 kDa Membrane Protein at the following dilutions.		
		1:10	1:100	1:1000
WN Kunjin Polio	6.5 ± 0.80	4.5 ± 0.45	0.6 ± 0.50	
	4.0 ± 0.35	2.8 ± 0.60	0.35 ± 0.20	
	0.85 ± 0.90	0.90 ± 0.55	0.75 ± 0.20	

[00200] The entry of West Nile and kunjin virus was strongly inhibited, while preimmune sera did not cause any inhibition. In contrast, the entry of poliovirus, a non-related picornavirus, was not affected in the presence of the 105-kDa protein antibody.

[00201] These results therefore strongly support the conclusion that the 105-kDa glycoprotein is the receptor for West Nile virus. Additionally, since the anti-105-kDa membrane protein antibodies were also effective in blocking the entry of the flavivirus kunjin virus, other flaviviruses of the Japanese encephalitis serocomplex subgroup, such as St. Louis encephalitis, Murray Valley encephalitis, kunjin viruses, dengue virus 1, dengue virus 2, dengue virus 3 and dengue virus 4, might also utilize this 105-kDa cell receptor protein for entry into host cells.

Example 12: Location of the 105-kda cell receptor protein in vertebrates and organs

[00202] Detection of the cell receptor protein from plasma membrane extracts from cells of several species, including vertebrates, utilizing a West Nile virus antibody and/or the cell receptor protein antibody is expected. Exemplary species comprise crows, horses, mice and humans, to determine if the cell receptor protein is present in these groups and if it can bind a flavivirus. This is shown by providing membrane proteins, loaded into different gel lanes, separated by SDS-PAGE and transferred onto nitrocellulose membrane. The nitrocellulose membranes are incubated sequentially with

purified West Nile virus, rabbit polyclonal mono-specific antibody against the viral envelope protein and then detected by secondary anti-rabbit antibodies conjugated with alkaline phosphatase with addition of substrate (NBT), for example.

[00203] The nitrocellulose membranes can be stripped and re-probed with the 105 kDa protein antibody. Exemplary incubation of separated membrane proteins with preimmune serum and antibodies at a dilution of 1:500 for crows, horses, mice and humans is contemplated, as well as use of Goat anti-mouse IgG conjugated with alkaline phosphatase, at a dilution of 1:2000. The antibody binding generated will be highly specific for 105-kDa membrane protein in a range of species. Antibody binding found in all species indicates that the cell receptor protein is present in a wide range of vertebrates. This would account for the wide pathogenicity of the West Nile virus among vertebrates.

Example 13: Peptide sequencing of the 105-kda cell receptor protein

[00204] Peptide sequencing of the 105 KDa membrane-associated glycoprotein was carried out to determine the identity of the glycoprotein following the procedure disclosed in Sagara et al, 1998

[00205] The amino acidic sequences reported in the sequence listing as SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, have been obtained as a result. These sequences closely match to those of the integrin superfamily after performing a database protein homology search in the databases Entrez Protein and NCBI, following procedures known to the person skilled in the art. Therefore, the peptide sequencing shows that the 105 KDa glycoprotein belongs to the integrin superfamily.

Example 14: 105-kda cell receptor protein identity

[00206] To determine the specific integrin molecule(s) or its (their) subunit of the integrin superfamily that mediate binding and entry of West Nile Virus, Vero cells were pre-incubated with a panel of functional blocking antibodies against integrins ($\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, αV , $\beta 1$, $\beta 2$, $\beta 3$, $\beta 4$, $\beta 5$ - Chemicon USA and Santa Cruz Biotech, USA) following procedures disclosed in Sagara et al 1998 at 4°C (to determine virus binding) or 37°C (to determine virus entry). Antibodies from different In particula antibodies from

different companies has been used to ensure reliability of results on inhibitory effect on West Nile virus binding to Vero cells.

[00207] Radioactive labeled West Nile Virus were then added to the treated cells and further incubated for 1 h at 4 °C or 37 °C. Excess and unbound particles were inactivated in acid glycine buffer (pH3) and removed by washing with PBS for three times. Results concerning virus binding are shown in Figure 11A. Results concerning virus entry are shown in Figure 11B. A significant inhibition of the entry of the virus is shown by treatment of antibodies against all the integrins subunits tested. Antibodies against integrin α V β 3 and its individual subunits (α V and β 3) showed the highest inhibition of West Nile virus binding and entry. Anti α V β 5 integrin antibodies also show some extent of West Nile virus binding and entry. In particular α V β 5 is also capable of blocking the binding of West Nile virus and much lesser effect on the entry of West Nile Virus, showing that integrin alpha V is functioning as the specific binding molecule for WNV.

[00208] This experiment is repeated with another closely related flavivirus, Japanese Encephalitis Virus using all the anti integrin antibodies at 25 μ g/ml. Results are shown in Figure 12. Antibodies against integrin subunits (α V and β 3) and integrin α V β 3 strongly inhibit the entry of Japanese Encephalitis Virus into Vero cells.

[00209] Therefore, the results obtained show that α V β 3 is the receptor molecule for both West Nile and Japanese Encephalitis virus and that both the integrin subunits of α V and β 3 are required in binding to West Nile and Japanese Encephalitis virus.

Example 15: Role of divalent cations in the binding of WNV and JEV

[00210] Confluent Vero cells were first washed with phosphate buffered saline (pH 7.4) and added with fresh culture medium M199 with 1% FCS containing EDTA (3-12 mM). The cells were incubated with EDTA for 2 hrs at 37 °C. After the incubation period, Vero cells were added with radiolabelled WNV particles and assess for its entry into Vero cells.

[00211] Divalent cations (Ca^{2+}) have been shown to be involved in the specific binding of physiological ligands to integrin. The possible requirement of divalent cations for binding of WNV to integrins was investigated by using EDTA (divalent cations

chelators). Vero cells were treated with EDTA at a series of concentration that has been shown to inhibit the binding of physiological ligands to integrin. Results are shown in Figure 13. Vero cells treated with EDTA did not block the binding and subsequent entry of West Nile virus. Therefore, removal of divalent cations from the culture environment did not have any effect on the entry of WNV into Vero cells.

Example 16: Competitive physiological ligand binding assay

[00212] Competitive physiological ligand binding assay with fibronectin, vitronectin, heparin, chondroitin sulphate, laminin and the peptides RGD1 and RGE1 reported in the sequence listing as SEQ ID NO: 6 and SEQ ID NO:7 was carried out. In particular Vero cells were incubated with different concentrations of ECM proteins, RGD peptides, or anti-integrin antibodies at 4°C or 37 °C for 1 hr, washed, incubated with 50 µl of radiolabeled WNV, JEV at 37°C for 1 hr and assessed for virus entry.

[00213] Results are shown in Figure 14. Binding of fibronectin vitronectin, laminin, and RGD peptide to cell surface has results only in partial inhibition of west Nile virus infection. At the same time, the partial inhibition by RGD peptide and physiological ligand (vitronectin) for integrin α V β 3 may propose that the binding of WNV to integrin may not be highly dependent on the usage of RGD motif on envelope protein of virus.

[00214] Previous studies have shown that many ligands and viruses can bind to integrin independently in the absence of RGD motif. Furthermore, site-directed mutagenesis of RGD motif in Murray Valley virus (flavivirus-JE serogroup) also shows consistent results that binding of Murray Valley virus to cells is independent of RGD motif on its envelope protein.

Example 17: Distribution and localization of the 105 KDa glycoprotein

[00215] To compare and confirm the results of experiments reported in examples 14 to 16, cell surface staining by immunofluorescence assay was carried out with antibodies against 105 KDa membrane protein and integrin α V β 3, respectively. Vero cells were fixed with methanol and processed for immunofluorescence staining. For immunofluorescence microscopy, cell monolayers were grown on cover slips and fixed with cold absolute methanol for 10 min. Subsequent procedure is similar to that described in Chu & Ng (2002). Antibodies against integrin α V β 3 and 105 KDa plasma

membrane glycoprotein were used as the primary antibodies, respectively. Primary antibodies and specific secondary antibodies conjugated with FITC were added subsequently. The primary antibody utilized was an anti-105-kDa polyclonal antibody (1:100) and anti-integrin alphaV beta 3 monoclonal antibody (1:500), at indicated dilutions. The fluorochrome used was an FITC conjugated secondary antibody. The specimens were viewed with Olympus IX81 using oil immersion objectives.

[00216] Results are shown in Figure 15 A and B, respectively. In particular, integrins $\alpha V\beta 3$ are distributed along the plasma membrane and focal adhesion through the cytoplasm (see Figure 15A). Similar distribution patterns of the 105KDa glycoprotein were also observed when compared to integrin $\alpha V\beta 3$ shown in Figure 15A. Thus, a similar distribution pattern between the 105 KDa glycoprotein and integrin $\alpha V\beta 3$ is observed.

[00217] Therefore, these data show that the WNV virus binding 105 KDa plasma membrane protein is the integrin $\alpha V\beta 3$.

Example 18: Gene knockout and down regulation of integrin $\alpha V\beta 3$ in Vero cells

[00218] To further confirm that integrin subunits αV and $\beta 3$ are the receptor molecules for West Nile Virus, gene knockout by means of RNA interfering was carried out. Ten short gene sequences from the full length integrin αV gene sequence (SEQ ID NO: 8) and twelve short gene sequences from the full length integrin $\beta 3$ gene sequence (SEQ ID NO:10) were selected and ligated into BamHI and HindIII digested pSilencer 3.0. In particular the following integrin sequences reported on the sequence listing as from SEQ ID NO: 12 to SEQ ID NO 15, were used: Integrin alpha V1 (SEQ ID NO: 12), Integrin alpha V2: (SEQ ID NO: 13); Integrin beta 31 (SEQ ID NO: 14); Integrin beta 32: (SEQ ID NO: 15). All twenty-two clones were selected and sequenced to verify in-frame insertion. All clones were then transfected into Vero cells and screened for down regulation of integrin expression.

[00219] In particular, plasmid constructs (psilencer 3.0-H1, Ambion, USA) containing different regions of the integrin alpha V beta 3 subunits (shown below) were constructed. Transfections were performed using Lipofectamine PLUS reagents from Invitrogen (USA) as specified by the manufacturer. In brief, Vero cells were grown on

coverslips in 24-wells tissue culture plate until 75% confluency. 1 to 5 μ g of the respective constructs was complexed with 4 μ l of PLUS reagent in 25 μ l of OPTI-MEM medium (GIBCO) for 15 min at room temperature. The mixture was then added to 25 μ l of OPTI-MEM containing 2 μ l of lipofectamine. After incubation for another 15 min, the DNA-liposome complexes were added to the cells. Following incubation for 3 h at 37 °C, 1 ml of complete growth medium was added and incubated for another 24 hrs before virus entry assay was carried out. The down regulation of integrin was checked by immunofluorescence assay using antibodies against integrin alphaV and beta 3.

[00220] The transfection efficiency was determined to be approximately 35%. Results are shown in Figure 16. Control formed by immunofluorescence staining of integrin α V and β 3 on Vero cells using anti integrin α V and β 3 antibodies is shown in Figures 16A and 16C. Those Figures respectively show that integrins α V and β 3 are both mainly distributed at the cell surface and the focal adhesion junction. Vero cells transfected with pSilencer-siRNA integrin α V or integrin β 3 shown in Figure 16B and Figure 16D respectively, show down regulation of integrin α V and β 3 on Vero cells.

[00221] In particular, a number of these clones can strongly down-regulate the expression of integrin α V or β 3 by 80%. These integrin down-regulated clones were selected for WNV virus entry study. Results are shown in Figure 17. The down-regulation of either integrin α V or β 3 strongly inhibited the entry of West Nile Virus as compared to internal control of GADPH.

Example 19: ATPases Antibody Blocking Virus Entry Assays

[00222] Antibody Blocking Virus Entry Assays (ABVEA) performed using antibodies against various plasma membrane-associated proteins have shown significant inhibition of virus entry of blockage of ATPases (data not shown). To investigate the role of an ATPase as co-receptor of the West Nile Virus, a further series of ABVEA was performed using antibodies raised against plasma membrane related ATPases.

[00223] In particular Vero cells were grown in 96 wells microtitre plates till confluent. Cells were washed thrice with PBS and incubated with antibodies against ATPase beta subunit, ATPase alpha subunit, calcium dihydropyridine receptor alpha, calcium dihydropyridine receptor beta, VATPase E and VATPase for 1 hr at 37 °C. Excess

antibodies were removed by washing thrice with PBS. 50 μ l of radiolabeled WNV were added and incubated for another 1 hr at 37 °C. Excess virus were then inactivated and washed with acid glycine buffer (pH 2.8). Penetrated virus were then determined.

[00224] Results are shown in Figure 18. The blocking of both plasma membrane associated ATPases and vacuolar ATPases with their respective antibodies seem to exert an inhibitory effect on the entry process of WNV. In general, ATPases are required to generate energy for many cellular activities across the plasma membrane. Hence, ATPases may act as co-receptor for WNV binding and providing the necessary energy for the endocytosis process of WNV.

Example 20: Neurotensin Antibody Blocking Virus Entry Assays

[00225] By using human brain cDNA library screening for interacting partners with WNV envelope protein in Yeast-2 hybrid system (not shown), a neurotensin receptor was obtained after several rounds of stringent selection. DNA sequence coding for neurotensin receptor is reported in the sequence listing as SEQ ID NO: 16, the amino acid sequence reported as SEQ ID NO:17.

[00226] ABVEA were performed with antibodies against the neurotensin receptor in A172 neuroblastoma cells and in Vero cells as a control.

[00227] In particular Vero cells were grown in 96 wells microtitre plates till confluent. Cells were washed thrice with PBS and incubated with antibodies against neurotensin receptor for 1 hr at 37 °C. Excess antibodies were removed by washing thrice with PBS. 50 μ l of radiolabeled WNV were added and incubated for another 1 hr at 37 °C. Excess virus were then inactivated and washed with acid glycine buffer (pH 2.8). Penetrated virus were then determined.

[00228] Results are shown in Figure 19. Both A172 and Vero cells were pre-incubated with different concentrations of antibodies against neurotensin receptors and followed by incubation with radiolabeled WNV. Entry of WNV is significantly inhibited by anti-neurotensin receptor antibodies in A172 cells but not in Vero cells.

[00229] These results have been confirmed by WNV competitive binding assays of neurotensin receptor with its natural ligand. Results of such assays are shown in Figure 20. Neurotensin (natural ligand) competitively blocked entry of WNV into A172 cells.

A172 cells were pretreated with neurotensin at a different concentration before incubation of cells with radiolabeled WNV. Entry of WNV is blocked in a dosage dependent manner.

Example 21: Effect of neurotensin down regulation to West Nile Virus entry in A172 cells

[00230] Work has been carried out to knockout the expression of neurotensin receptor in A172 cells and assess for West Nile virus entry. Three short gene sequences from the full length neurotensin receptor sequence were selected and ligated in BamHI and HindII digested pSilencer 3.0 (not shown). In particular the sequence reported in the sequence listing as SEQ ID NO: 18 was used.

[00231] Plasmid constructs (psilencer 3.0-H1, Ambion, USA) containing the neurotensin receptor sequence reported in the sequence listing as SEQ ID NO: 18 was transfected into A172 cells. Transfections were performed using Lipofectamine PLUS reagents from Invitrogen (USA) as specified by the manufacturer. In brief, Vero cells were grown on coverslips in 24-wells tissue culture plate until 75% confluence. 1 to 5 ug of the respective constructs was complexed with 4 μ l of PLUS reagent in 25 μ l of OPTI-MEM medium (GIBCO) for 15 min at room temperature. The mixture was then added to 25 μ l of OPTI-MEM containing 2 μ l of lipofectamine. After incubation for another 15 min, the DNA-liposome complexes were added to the cells. Following incubation for 3 h at 37 °C, 1 ml of complete growth medium was added and incubated for another 24 hrs before virus entry assay was carried out.

[00232] Clones were selected and sequenced to verify in frame insertion (not shown). All clones were then transfected into A172 cells and screened for down-regulation of neurotensin receptor expression. The down regulation of neurotensin receptor was checked by immunofluorescence assay using antibodies against neurotensin receptor. To demonstrate expression and localization of the receptor immunofluorescence assay with the antineurotensin receptor, antibodies were used. Results are shown in Figure 21. A172 cells transfected with pSilencer-siRNA expressing siRNA against the neurotensin receptor showed a down regulation of plasma membrane neurotensin receptor expression (B). No down regulation was observed instead in the control, where

in absence of transfection with pSilencer-siRNA neurotensin receptor is expressed predominantly on the plasma membrane and within cytoplasmic vesicles (A).

Example 22: West Nile Virus attachment domain

[00233] West Nile virus envelope domain III (350-390) was cloned into *E. coli* expression vector pET16b (Novogen, USA) and expressed as His-tagged fusion protein. The DIII protein was expressed as a soluble protein and was purified through a nickel column. The purified DIII protein was separated by 10% SDS PAGE and followed by transferring to nitrocellulose membrane. The recombinant DIII is detected with monoclonal antibodies against E protein of WNV and anti-His antibodies. The monoclonal anti-E protein and anti-His antibodies were used at a concentration of 1:500 and 1: 200 respectively. The secondary antibodies conjugated with alkaline phosphatase were added subsequently. Detection of DIII protein was carried out by adding the substrate (nitroblue tetrazolium) to the blot.

[00234] Vero cells were first incubated with different concentration (5 to 100 µg/ml) of DIII protein or BSA for 30 min at 37°C. Excess or unbound protein is removed by washing thrice with PBS. Radiolabeled WNV or Dengue virus (250 plaque forming unit, PFU) is added and incubated for 1 h at 37°C. Virus entry into Vero cells was determined by radioactive counts from a scintillation counter.

[00235] The production of murine polyclonal antibodies against West Nile virus DIII protein was carried out as previously described by Chu and Ng, 2003. The pool sera from 6 DIII protein immunized Balb/c mice were diluted in a series of concentration 1:2 to 1:8192. Equal volume (50 µl) of anti-DIII antibodies and WNV (500 PFU) were incubated for 1 h before overlaying onto Vero cells monolayer. Excess or unbound virus-antibody complexes were removed by washing thrice with PBS. Plaques were stained with crystal violet after 4 days of incubation at 37°C. Virus diluent was used as a control for anti-DIII antibodies.

[00236] The portion of West Nile Virus coding for the domain III is reported herein as SEQ ID NO: 20, [SEQ ID NO: 20: cggaaattcag cttcaactgt ttaggaatga gcaacaggga cttcctggag ggagtgtctg gagctacatg ggttgatctg gtactggaag gagacagttg tgtgaccata atgtcaaaag acaagccaac cattgatgtc aaaatgatga acatggaagc agctaattctc gcagatgtgc gtagctactg ctacttagct tcggtcagtg atctgtcaac aaaagccgcg tgtccaacca tgggtgaagc

tcacaacgag aaaagagccg accctgcctt tgtttcaag caaggcgtcg tagacagagg atggggaat
ggatgcggac tgtttggaaa ggggagcatt gacacatgtg caaagttgc ctgtacaacc aaggcaactg
gttggattat ccagaaggaa aacatcaagt acgagggtgc catatttgc catggcccg a cgactgtcga
atcacatggc aattattcaa cacagatagg ggctacccaa gcaggaaggt tcagcataac tccatcgca
ccatcctaca cgctgaagtt gggtgagtt ggtgaggtca cagttgactg tgagccacgg tcaggaatag
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tttggaggaa tgcctggat cacacagggg cttctggag ctcttctgtt gtggatggga attaacgccc
gtgacaggc aattgctatg acgttccttgc ggttggagg agtcttgctc ttcccttcgg tcaacgtcca
tgctggatcc]. This sequence encodes for domain III of the Envelope protein according to
the well known genetic code. A person skilled in the art can easily identify the coding
portions inside the sequence as well as the amino acid sequence encoded by SEQ ID
NO: 21.

[00237] The amino acid sequence of domain III coded by the sequence reported as SEQ ID NO: 20 is designated as SEQ ID NO: 21. A soluble form of recombinant domain III from 350 to 390 of the envelope protein of West Nile virus envelope protein (E protein) was cloned into E. Coli expression vector pET16b (Novogen, USA) and expressed as His-tagged fusion protein. The protein had comprised a sequence reported in the sequence listing as SEQ ID NO: 19. The DIII protein was able to be expressed as a soluble protein. Recombinant DIII protein was separated by 10%SDS PAGE and followed by transferring to nitrocellulose membrane. The recombinant DIII is detected with monoclonal antibodies against E protein of WNV and anti-His antibodies. The results are shown in Figure 22.

[00238] Also polyclonal antibodies (not shown) against West Nile virus E-protein were able to detect the expressed recombinant E domain III (Figure 22).

[00239] Subsequently, Vero cells were first incubated with different concentration of DIII protein or BSA. Radiolabeled WNV or Dengue virus is added and assay for virus entry. Results are shown in Figure 23. Entry of WNV is significantly blocked in the presence of DIII protein while BSA did not have any effect on the entry of WNV. Recombinant WNV envelope DIII protein can also slightly block the entry of Dengue virus at high concentration used. Therefore, recombinant E domain III was also able to competitively inhibit the binding of West Nile virus in a dosage dependent manner.

[00240] In addition, murine polyclonal antibodies were produced against the recombinant domain III (Figure 24). A single 13 KDA protein band (DIII protein) was detected by the murine polyclonal antibodies.

[00241] These murine polyclonal antibodies were used in plaque neutralization assay of WNV. Recombinant DIII is expressed, purified and injected into 6 Balb/c mice. pool sera were obtained and diluted in a series of concentration as shown in the graph below. Equal volume of anti-DIII antibodies and WNV (500 PFU) were incubated for 1 h before overlaying onto Vero cells monolayer. Plaques were stained with crystal violet. Virus diluent was used as a control for anti-DIII antibodies. Results shown in Figure 25 demonstrate that murine polyclonal antibodies against DIII protein are capable of neutralizing the West Nile virus

[00242] Together, these data define that domain III of West Nile virus E protein is responsible for binding to the surface of the cells.

[00243] The disclosures of each and every publication and reference cited herein are incorporated herein by reference in their entirety.

[0001] The present disclosure has been explained with reference to specific embodiments. Other embodiments will be apparent to those of ordinary skill in the art in view of the foregoing description. The scope of protection of the present disclosure is defined by the appended claims.

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